

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

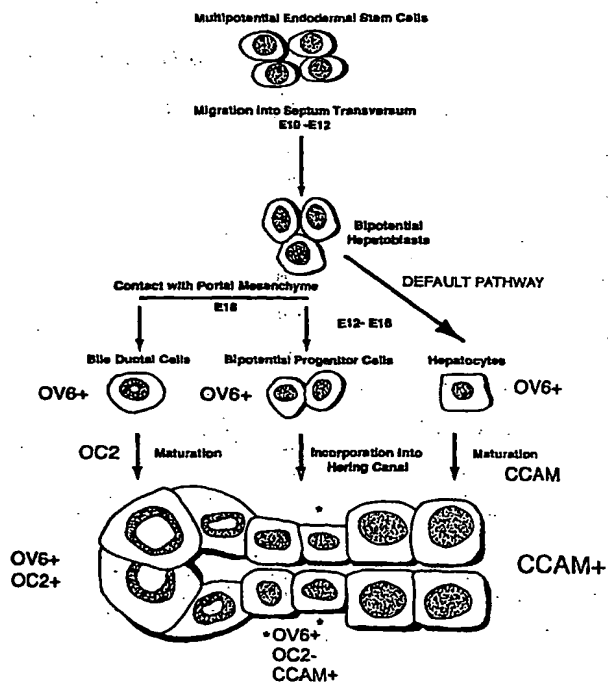
(51) International Patent Classification ⁷ : C12N 5/06, 5/16, A61M 1/14		A1	(11) International Publication Number: WO 00/03001
			(43) International Publication Date: 20 January 2000 (20.01.00)
(21) International Application Number: PCT/US99/15625 (22) International Filing Date: 8 July 1999 (08.07.99) (30) Priority Data: 09/113,774 10 July 1998 (10.07.98) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 09/113,774 (CON) Filed on 10 July 1998 (10.07.98) (71) Applicant (for all designated States except US): RHODE ISLAND HOSPITAL [US/US]; 593 Eddy Street, Providence, RI 02903 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): FARIS, Ronald, A. [US/US]; 214 Sumter Street, Providence, RI 02907 (US). (74) Agent: BEATTIE, Ingrid, A.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	

(54) Title: LIVER STEM CELL

(57) Abstract

The invention provides a primary liver stem cell and a cell doublet consisting of a hepatocyte and the stem cell, both of which are derived from normal liver tissue. Methods of isolating the cells, genetically altering the cells, and using the cells for transplantation are also within the invention.

ANTIGENIC PATHWAY OF LIVER DEVELOPMENT



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
UG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
HJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

LIVER STEM CELLBackground of the Invention

5 The invention relates to the cell-mediated treatment of liver disease.

 Patients who suffer from severe, irreversible liver disease for which other medical and surgical treatments have failed are often candidates for liver
10 transplantation. In children, the most common indications are biliary atresia, a condition which leads to distortion of bile ducts and liver cirrhosis and genetically transmitted metabolic disorders which may lead to hepatic failure and/or cirrhosis. Adult
15 suffering from nonalcoholic or alcoholic cirrhosis as well as liver cancer may be candidates for transplantation.

 The existence of an adult liver stem cells remains the subject of controversy. However, stem cells which
20 differentiate into functional mature hepatocytes to reconstitute a diseased liver may be an alternative approach to whole organ transplantation for the treatment of certain liver diseases.

Summary of the Invention

25 The invention provides primary liver stem cells which can be used to treat degenerative liver diseases or inherited deficiencies of liver function, e.g., those characterized by production of a mutant protein or by the misregulation of protein expression that results in liver
30 dysfunction. The stem cells may be multipotential, e.g., the cells can differentiate into hepatocytes or bile ductal cells, or they may be precommitted to differentiating into hepatocytes. The invention also provides a method for isolating and utilizing stem cells
35 and cell doublets not only for hepatic transplantation

- 2 -

but also for gene therapy and as the biological component of liver assist devices.

Accordingly, the invention includes a primary liver cell cluster containing a liver stem cell and a hepatocyte or an isolated primary liver stem cell. By "stem cell" is meant an undifferentiated cell that differentiates into a mature functional hepatocyte or bile duct cell. "Facultative" liver stem cells (FLSC) require a stimulus to proliferate. For example, FLSC proliferate in response to stress or injury such as exposure to a carcinogen. Stem cells are not fully differentiated and retain the ability to proliferate under conditions which differentiated liver cells normally do not proliferate, e.g., following exposure to chemical carcinogens. By "cell cluster" is meant a group of at least two associated cells. Preferably, the cluster contains less than 10 cells, more preferably less than 5 cells. Typically, the isolated cell clusters contain between 2 and 5 cells per cluster. Most preferably, the cluster is a cell doublet, i.e., a cluster containing two cells, or a cell triplet, i.e., a cluster containing three cells. Within the cluster, at least two of the cells are joined by a desmosomal junction. By "normal" liver tissue is meant tissue that is noncancerous and uninfected by pathogenic microorganisms.

The stem cell is distinguished from and can be separated from other undifferentiated or partially differentiated liver cells, e.g., oval cells, by virtue of its association with a hepatocyte. The hepatocyte and stem cell of the doublet are joined by desmosomal junctions. The stem cell is preferably a pre-oval cell and is distinguished from oval cells by the tight association with a hepatocyte and lack of detectable expression of an oval cell marker such as OC2.

- 3 -

Preferably the stem cell expresses the marker OV6. The stem cell may also express a bile duct cell marker such as a cytokeratin, e.g., cytokeratin 19. Other cell surface markers such as an extracellular matrix marker
5 such as laminin, a desmosomal glycoprotein such as desmoplakin I, a liver cell adhesion molecule such as cell-cell adhesion molecule (CCAM), a carcinoembryonic antigen (CEA), dipeptidyl peptidase-4, a bile duct marker on oval cells such as γ -glutamyl transpeptidase (γ GT),
10 Very Late After Activation (VLA)-2, VLA-3, VLA-5, or VLA-6 may also be expressed.

Isolated liver cell clusters and isolated stem cells may be obtained from fetal, pediatric, or adult liver tissue. Preferably, the cells are obtained from
15 adult liver tissue rather than fetal tissue. The cells may differentiate into mature functional hepatocytes or mature bile duct cells. Preferably, the stem cells differentiate into mature functional hepatocytes, i.e., hepatocytes characterized by liver-specific
20 differentiated metabolic functions, e.g., the expression of albumin, CCAM, glucose-6-phosphatase, α_1 -antitrypsin, or P450 enzyme activity.

The stem cells may be genetically-altered by the introduction of heterologous DNA. A genetically-altered
25 stem cell is one into which has been introduced, by means of recombinant DNA techniques, a nucleic acid encoding a polypeptide. The DNA is separated from the 5' and 3' coding sequences with which it is immediately contiguous in the naturally occurring genome of an organism, e.g.,
30 the DNA may be a cDNA or fragment thereof. In some cases, the underlying defect of a pathological state is a mutation in DNA encoding a protein such as a metabolic protein. Preferably, the polypeptide encoded by the heterologous DNA lacks a mutation associated with a
35 pathological state. In other cases, a pathological state

- 4 -

is associated with a decrease in expression of a protein. A genetically-altered stem cell may contain DNA encoding such a protein under the control of a promoter that directs strong expression of the recombinant protein.

- 5 Such cells, when transplanted into an individual suffering from abnormally low expression of the protein, produce high levels of the protein to confer a therapeutic benefit. For example, the stem cell contains heterologous DNA encoding a metabolic protein such as
- 10 ornithine transcarbamylase, arginosuccinate synthetase, glutamine synthetase, glycogen synthetase, glucose-6-phosphatase, succinate dehydrogenase, glucokinase, pyruvate kinase, acetyl CoA carboxylase, fatty acid synthetase, alanine aminotransferase, glutamate
- 15 dehydrogenase, ferritin, low density lipoprotein (LDL) receptor, P450 enzymes, or alcohol dehydrogenase. Alternatively, the cell may contain DNA encoding a secreted plasma protein such as albumin, transferrin, complement component C3, α_2 -macroglobulin, fibrinogen,
- 20 Factor XIII:C, Factor IX, or α_1 -antitrypsin.

The term "isolated" used in reference to a single cell or cell cluster, e.g., a stem cell or stem cell-hepatocyte triplet or doublet, means that the cell or cell cluster is substantially free of other cell types or

25 cellular material with which it naturally occurs in the liver. A sample of stem cells or doublets is "substantially pure" when it is at least 60% of the cell population. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at

30 least 99%, of the cell population. Purity can be measured by any appropriate standard method, for example, by fluorescence-activated cell sorting (FACS).

The invention includes a method of obtaining a sample of liver stem cells by (a) isolating a cell

35 doublet from normal liver tissue, (b) dissociating the

- 5 -

stem cell from the hepatocyte, and (c) removing the hepatocyte from the doublet to yield a sample of liver stem cells. The method optionally includes selecting for the expression of other cell markers, such as desmoplakin, OV6, cytokeratin 19, laminin, or CCAM. Preferably, the method includes a step of selecting for cells which lack oval cell marker OC2 expression to enrich for the desired stem cells, i.e., selecting against contaminating bile duct cells which express OC2.

10 A method of hepatic transplantation is also within the invention. A patient in need of a liver transplant such as one suffering from degenerative liver disease, cancer, or a metabolic disease, is treated by transplanting into the patient a stem cell or stem cell-hepatocyte doublet. To treat an inherited or acquired genetic or metabolic disease, a genetically-altered stem cell (singly or paired with a hepatocyte) is transplanted. For example, the stem cell may be transfected with DNA encoding Factor VIII:C, Factor IX, α_1 antitrypsin, or low density lipoprotein receptor useful for treating human diseases such as hemophilia A and B, α_1 antitrypsin deficiency, and familial hypercholesterolemia, respectively. Genetically-altered stem cells are useful as an in vivo recombinant protein delivery system and have the advantage of being capable of immortality (and thus, greater long-term survival) compared to differentiated cells, i.e., stem cells are capable of giving rise to differentiated progeny but retain the capacity for self-renewal.

25 The cells of the invention are also useful as the biological component of a perfusion device or as a source of functional differentiated hepatocytes which can then be used as the biological component of a perfusion device such as a liver assist device (LAD) or bioreactor.

35 Contact of a patient-derived bodily fluid with the such

- 6 -

hepatocytes results in detoxification of the bodily fluid for subsequently return to the patient.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Brief Description of the Drawing

Fig. 1 is a diagram showing development of a liver stem cell.

Fig. 2 is a photomicrograph of a liver cell doublet derived from normal adult rat liver tissue. The stem cell (indicated by an arrow) shows positive labelling with an antibody specific for the cell marker OV6. The stem cell and hepatocyte of the doublet are joined by desmosomal junctions.

Fig. 3 is a photomicrograph of a liver cell doublet derived from normal adult human liver tissue. The stem cell (indicated by an arrow) shows positive labelling with an antibody specific for the cell marker CK19. The stem cell and hepatocyte of the doublet are tightly joined by desmosomal junctions.

- 7 -

Figs. 4A and 4B are photomicrographs of stem cell-hepatocyte cell doublets derived from rat liver tissue showing the tight association between the hepatocyte and the stem cell. The two cells of the cell doublet are joined by desmosomal junctions as demonstrated by positive staining with an antibody specific for desmoplakin, a component of the desmosomal junction. Figs. 4A and 4B are photographs of the same cells in the same microscopic field. Fig. 4A shows CK19 staining, and Fig. 4B shows desmoplakin staining. Simultaneous double label immunofluorescence analysis shows that the stem cell (indicated with an arrow) expresses both CK19 and desmoplakin. The pattern of desmoplakin staining is continuous from the stem cell to the neighboring hepatocyte (denoted "H") indicating that the two cells were co-isolated and are joined by desmosomal junctions.

Figs. 5A and 5B are photomicrographs of two stem cells attached to a hepatocyte. The cell cluster was derived from rat liver tissue. Figs. 5A and 5B are photographs of the same cells in the same microscopic field. Fig. 5A is a view under phase microscopy, whereas Fig. 5B shows BrdU fluorescence.

Facultative liver stem cells

The existence of a liver stem cell has been disputed for decades. Following hepatic injury that impairs the replicative capacity of hepatocytes, a heterogenous population of small stem-like cells called oval cells arises in the liver. Oval cells are small cells of the liver with oval nuclei which proliferate in response to exposure to carcinogens. Oval cells do not exist in normal liver tissue, but arise after a stressful stimulus such as a mechanical injury or exposure to a carcinogen. Activation of a stem cell compartment of the liver gives rise to a population of oval cells. Such

- 8 -

oval cells are bipotential, i.e., they may differentiate into hepatocytes or bile ductal cells (Fig. 1).

Most attempts to isolate stem cells have been based on the expression of oval cell antigens, e.g., OV6, OC2, and OC3, by putative stem cells. However, these antigens may also be present on other liver cell types, e.g., bile duct cells or mesothelial cells, resulting in contamination of a preparation of putative stem cells with these other cell types. The invention identifies a novel phenotypic profile and solves the contamination problem by requiring isolation of a specific type of liver cell cluster, i.e., one characterized by a hepatocyte joined to a small non-hepatocytic cell via desmosomal junctions. Further isolation measures may include selection for expression of bile duct cell-specific antigens.

Oval cells are the progeny of the stem cells described herein. Although the stem cells of the invention share many cell markers with other liver cells, they are distinguished from oval cells and other putative stem cells by virtue of their tight association with a hepatocyte. This distinguishing characteristic allows identification and purification of a unique stem cell that upon proliferation gives rise to oval cells which differentiate into cells of hepatic lineage (rather than biliary lineage). The isolated stem cells (or cell clusters) can be used to repopulate a damaged liver, for gene therapy, and as the biological component of a liver assist device.

Preparation of liver stem cells and liver cell clusters

Liver tissue is enzymatically digested to dissociate cells from connective tissue while preserving the integrity of stem cell-hepatocyte clusters. In vivo, the stem cells reside in a unique niche of the liver, i.e., the canals of Hering, and stem cells derived from

- 9 -

this niche are identified by their expression of one or more bile duct cell markers. Previous cell isolation protocols yielded single cell suspensions, whereas the method of the invention provides for isolation of cell clusters. Participation in a cell cluster represents a reliable distinguishing character of a pre-oval cell stem cell and is the only known marker of this cell type. Following enzymatic dissociation of the liver, the cell suspension is enriched for periportal hepatocytes associated with the biliary tree, and the cell suspension is subjected to enrichment for cell clusters, e.g., cell doublets, which contain a cell that expresses bile ductal antigens. For example, a suspension of rodent or human liver cells is subjected to selection for stem cells or clusters which express the marker CK19.

Mammalian organ donors may be used to provide liver tissue from which stem cells and doublets are isolated. For example, tissue is obtained from a rodent such as a mouse or rat, a dog, a baboon, a pig, or a human. The tissue is obtained from a deceased donor, an aborted fetus, or from a living donor, e.g., from a needle biopsy, a small wedge biopsy, or a partial hepatectomy. In some cases, autologous cells may be obtained from a patient, manipulated in vitro, e.g., to introduce heterologous DNA, and returned to the patient. More typically, the cells are obtained from a heterologous donor. If the donor hepatocytes are heterologous, then donor-recipient histocompatibility is determined. Class I and class II histocompatibility antigens are determined and individuals closely matched immunologically to the patient are selected as donors. All donors are screened for the presence of transmissible viruses (e.g., human immunodeficiency virus, cytomegalovirus, hepatitis A/B). Suitable donors are those which are free from the tested infectious diseases.

- 10 -

Rat liver tissue and human liver tissue (obtained from cadavers) were used as sources of tissue for the preparation of stem cells and cell clusters. Male Fisher rats were obtained from Charles River. Reagents and 5 buffers are described below.

Table 1

	<u>Reagent</u>	<u>Vendor</u>	<u>Catalog #</u>	
	<u>Concentration</u>			
	DMEM-F12*	Gibco	12400-086	Stock
10	HBSS*	Gibco	61200-093	Stock
	CMF*	Gibco	21250-014	Stock
	HEPES	Sigma	H9136	0.1M
	CaCl ₂	Sigma	C7902	500mM
	BSA	Interger	3225-75	.4 mg/ml
15	STI	Gibco	17075-029	.1 mg/ml
	Collagenase Type IV	Worthington		60 units/ml
	BrdU	Sigma	B9285	150 mg/kg
	Percoll	Pharmacia	17089101	90%
20	Dynabeads	Dynal	110.05	1 x 10 ⁷ beads/ml
	2 AAF	Innovative Research of America	A-102	35 mg pellet (21 d
25	release)			

Preperfusion buffer was prepared by mixing CMF (475 ml) with 0.1 M Hepes (25 ml of stock in which 23.83 g Hepes was dissolved in 990 ml dH₂O, pH to 7.0, QS to 1 L and 30 filter sterilized). The digestion buffer used is formulated to liberate cells from the liver organ but to preserve the integrity of cell clusters (approximately 2-10 cells in size, optimally 2-3 cells in size) rather than to yield a suspension of single cells. Subjecting 35 liver tissue to the digestion buffer described does not yield a single cell suspension, but a mixture of single cells and cell clusters, e.g., doublets or triplets, and single cells. The clusters are then retrieved and the single cells discarded.

40 The digestion buffer (Digestion Buffer I) contains Collagenase Type IV (60 units/ml). Digestion Buffer I (100 ml) contains Preperfusion buffer (250 ml), CaCl₂ 500

- 11 -

mM 100x stock (2.5 ml), STI (0.025 g), and Collagenase Type IV (60 units/ml). Digestion Buffer II is a solution of 0.02 g of BSA in 50 ml of Digestion Buffer I. The cell suspension buffer contains HBSS (475 ml) and 0.1 M Hepes (25 ml). The cell washing buffer contains DMEM-F12 (500 ml) and BSA (5 g). CMF, HBSS and DMEM-F12 are typically oxygenated for 5 minutes prior to adding other reagents. The pH of the preperfusion, suspension and washing buffers is adjusted to 7.2-7.3, the digestion buffer to 7.4-7.5. All buffers are filtered using 0.2 micron filter. The preperfusion, digestion and suspension buffers are used at 37° C, while the washing buffer is kept ice cold.

Male Fisher rats weighing between 115 g - 170 g were anesthetized with Xylazine (10 mg/kg) and Ketamine (50 mg/kg). The inferior vena cava was cannulated in the vicinity of the right renal vein, the aorta tied off, and the portal vein cut. The liver was perfused with preperfusion buffer at a flow rate of 20 ml/min. for approximately 4-5 mins., until the blood of the liver was cleared. Perfusion was then continued using Digestion Buffer at 30 ml/min. for approximately 6-8 min. The liver was excised, minced, and placed in a spinner flask with 100 ml of suspension buffer. The flask was placed in a 37° C incubator on a stirring plate for 40-50 minutes. The combined suspension was sequentially filtered through a 230 micron steel mesh filter, and a 60 micron nylon mesh filter. The remnant remaining on the filters was washed off and placed in a 25 ml flask with 10 ml of digestion buffer II. The flask was placed in a 37° C shaking water bath set to 160 shaker rate/min. After 20 minutes, the cell suspension was transferred to a 15 ml tube, and the suspension allowed to settle by gravity.

- 12 -

The supernatant and the remnant (settled material) were then separated. The supernatant was decanted and centrifuged at $80 \times g$ for 5 minutes. Fresh digestion buffer was added to the cells and placed back into the shaking water bath. The pellet remaining after the centrifugation was resuspended with washing buffer and kept on ice. If the cells appeared to be very adherent to the biliary tree, a solution of 1 mM EGTA dissolved in CMF was substituted for 5 minutes in place of a digestion step.

The cell suspension that was kept on ice, is then filtered through a 60 micron nylon mesh filter to remove large aggregates of cells and mixed with an equal volume of 90% Percoll and 10 % 10x DMEM-F12. This is then centrifuged at $300 \times g$ for 5 minutes. The pellet was resuspended in washing buffer, and centrifuged at $120 \times g$ for 5 minutes. The pellet is then resuspended in washing buffer.

An immunosubtraction step is first carried out to remove undesired cells, thereby enriching for desired stem cell-hepatocyte clusters. Dynabeads were conjugated to a mouse monoclonal antibody specific for rat bile duct and mesothelial cells (IgG_{2b}). The beads were added to the cell suspension, and incubated at $4^{\circ} C$ on a rotator for 10 minutes. The suspension was then placed on a magnet to remove antibody-positive cells; these cells were discarded. This step was repeated 3 additional times. The antibody-negative cells were subjected to more incubations with Dynabeads conjugated to an antibody specific for CCAM (e.g., anti-rat cell-CAM 105; Endogen); and antibody-positive cells with a stem cell attached (e.g., cell clusters such as doublets and triplets) were cultured and cytopinned.

Isolated cell clusters containing a stem cell and a hepatocyte are further processed to achieve a

- 13 -

population of isolated stem cells. For example, the sample of cells is trypsinized to dissociate the cell clusters, i.e., enzymatically disrupt the desmosomal junctions. Since hepatocytes are particularly sensitive to trypsin (or pronase), this step not only separates the cells but aids in removing the hepatocytes. The cell preparation is then subjected to further selection with antibodies specific for such cell markers as CK19 (Amersham), CCAM (Endogen), dipeptidyl peptidase-4 (Endogen) in combination with magnetic beads or FACS sorting to enrich for the desired stem cell. Antibodies to other markers such as γ GT, VLA-2, VLA-3, VLA-5, or VLA-6 CEA may also be used.

Figs. 2 and 3 show an isolated stem cell-hepatocyte doublet derived from a normal rat liver and a normal human liver, respectively. In each figure, the smaller of the two cells (indicated with an arrow) is the stem cell. The rat stem cell of the doublet is also OV6-positive; the human stem cell is CK19-positive (antibodies for CK19 bind to both rat and human stem cells). The stem cell and hepatocyte of the doublet are joined by desmosomal junctions (Figs. 4A and 4B).

Bioassay to Activate the Stem Cell Compartment

To confirm the identity of the stem cell isolated, proliferation of the stem cell compartment was carried out *in vivo* using a liver carcinogen and the stem cell-hepatocyte clusters isolated as described above. The proliferative capabilities were evaluated and the expression of cell markers measured.

Approximately 48 hours prior to stem cell isolation, male Fisher rats weighing between 115-170 g were anesthetized with metophane, and one pellet of the liver carcinogen 2-acetylaminofluorene (2-AAF) was placed in the peritoneal cavity of the animal. Alternatively, the carcinogen pellet was left in the animal for 2 weeks

- 14 -

prior to administration of a radioisotope (to measure cell proliferation) and subsequent sacrifice for retrieval of stem cells.

Cellular incorporation of bromouridine (BrdU) was used as a measure of cell proliferation. An hour after surgery, a dose of BrdU was dissolved in normal saline is injected intraperitoneally. Additional doses were given 4 and 22 hours later.

Rats were sacrificed and perfusion of the liver was commenced 2 hours after the last dose of BrdU was administered. Cell clusters were isolated as described above. Cell suspensions enriched for stem cell-hepatocyte doublets were further subjected to selection for doublets expressing CCAM and analyzed for expression of cell markers and proliferation.

Figs. 5A and 5B show a stem cell-hepatocyte triplet in which two OV6-positive stem cells are attached to a hepatocyte. One of the attached stem cells is strongly labelled with BrdU, indicating an actively proliferating cell. These results confirm that the small cell attached to a hepatocyte via desmosomal junctions is a liver stem cell.

Therapeutic Use

Stem cells and cell clusters are transplanted into individuals to treat a variety of pathological states including degenerative liver disease or disease characterized by production of a mutated protein or aberrant regulation of a non-mutated, i.e., normal, protein. The latter category of diseases include familial hypercholesterolemia, α_1 -antitrypsin deficiency, factor VIII deficiency (Hemophilia A) and factor IX deficiency (Hemophilia B) (see, e.g., Wilson et al., *Principles of Internal Medicine*, McGraw-Hill, N.Y., 1991)

Familial hypercholesterolemia is an autosomal dominant disorder in human patients caused by a deficiency of the receptor that mediates the uptake of

- 15 -

low density lipoprotein (see, e.g., Scriver et al. (eds) *The Metabolic Basis of Inherited Disease*, McGraw-Hill, NY, pp 1215-1250). The disease leads to elevated levels of serum cholesterol and premature development of coronary artery disease.

Alpha₁-antitrypsin deficiency is a hereditary disorder characterized by reduced serum levels of α_1 -antitrypsin, a protease inhibitor that provides the major defense for the lower respiratory tract against destructive proteases. Children homozygous for α_1 -antitrypsin deficiency will develop significant liver disease including neonatal hepatitis and progressive cirrhosis, and α_1 -antitrypsin deficiency adults can lead to asymptomatic cirrhosis.

Hemophilia A and hemophilia B are sex-linked inherited plasma coagulation disorders due to defects in factors VIII and factor IX, respectively. Previous treatments for hemophilia A involved administration of plasma products enriched for factor VIII. Treatment of affected patients with stem cells genetically-altered to produce recombinant clotting factors avoids the potential risk of exposing patients to viral contaminants, such as viral hepatitis and human immunodeficiency virus (HIV).

Cell transplantation

Stem cells or cell doublets (either as is or genetically-altered to produce a recombinant gene product) are introduced into an individual in need of a hepatic transplant or in need of the protein encoded by the genetically-altered cell. In addition to using the cells for treatment of degenerative liver disease, stem cells can be administered to cancer patients who have undergone chemotherapy to kill cancerous liver cells. Thus, after administration of the chemotherapeutic agent, the patient's liver can be "reseeded" with stem cells.

- 16 -

If the cells are derived from heterologous, concomitant immunosuppression therapy is typically administered, e.g., administration of the immunosuppressive agent cyclosporine or FK506.

- 5 Alternatively, the cells can be encapsulated in a membrane which permits exchange of fluids but prevents cell/cell contact. Transplantation of microencapsulated cells is known in the art, e.g., Balladur et al., 1995, Surgery 117:189-194; and Dixit et al., 1992, Cell
10 Transplantation 1:275-279.

The cells may be introduced directly to the liver, e.g., via the portal vein, or deposited within other locations throughout the body, e.g., the spleen, pancreas, or on microcarrier beads in the peritoneum.

- 15 For example, 10^2 to 10^9 cells are transplanted in a single procedure, and additional transplants are performed as required.

Differentiation of the stem cells is induced by contact with liver tissue, i.e., other hepatocytes or
20 cell matrix components. Optionally, a differentiating agent may be co-administered or subsequently administered to the patient to promote stem cell differentiation.

Genetically-altered stem cells

- Genetically-altered stem cells are useful to
25 produce therapeutic recombinant proteins *in vivo*. The stem cells are isolated from a donor (nonhuman or human), transfected or transformed with a recombinant gene in vitro, and transplanted into the recipient. The genetically-altered stem cells produce the desired
30 recombinant therapeutic protein in vivo leading to clinical improvement of the patient so treated.

Conventional gene transfer methods are used to introduce DNA into cells. The precise method used to introduce a replacement gene, e.g., clotting factor or
35 metabolic protein is not critical to the invention. For

- 17 -

example, physical methods for the introduction of DNA into cells include microinjection and electroporation. Chemical methods such as coprecipitation with calcium phosphate and incorporation of DNA into liposomes are also standard methods of introducing DNA into mammalian cells. DNA is introduced using standard vectors, such as those derived from murine and avian retroviruses (see, e.g., Gluzman et al., *Viral Vectors*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Standard recombinant DNA methods are well known in the art (see, e.g., Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989), and viral vectors for gene therapy have been developed and successfully used clinically (Rosenberg, et al., *N. Engl. J. Med*, 323:370 1990).

Liver Assist Devices

The stem cells, cell clusters, and progeny thereof are useful as the biological components of detoxification devices such as liver perfusion or liver assist devices.

A conventional liver assist device includes a rigid, plastic outer shell and hollow semi-permeable membrane fibers which are seeded with stem cells, cell doublets, or differentiated hepatocytes derived from the stem cells or cell clusters. Differentiation of stem cells is induced by contacting the cells with known differentiating factors, e.g., dimethylsulfoxide (DMSO), Vitamin A, sodium butyrate, or matrix components such as heparin sulfate.

The fibers may be treated with collagen, lectin, laminin, or fibronectin, for the attachment of cells or left untreated. Bodily fluid is perfused through the device for detoxification according to well known procedures and then returned to the patient.

Other embodiments are within the following claims.

- 18 -

What is claimed is:

1. An isolated liver cell cluster comprising a liver stem cell and a hepatocyte.
2. The liver cell cluster of claim 1, wherein
5 said liver cell cluster is a cell doublet.
3. The liver cell cluster of claim 1, wherein said hepatocyte and said stem cell are joined by a desmosomal junction.
4. The liver cell cluster of claim 1, wherein
10 said stem cell is a pre-oval cell.
5. The liver cell cluster of claim 1, wherein said stem cell expresses OV6.
6. The liver cell cluster of claim 1, wherein said stem cell expresses a bile duct cell marker.
- 15 7. The liver cell cluster of claim 6, wherein said bile ductal cell marker is a cytokeratin.
8. The liver cell cluster of claim 7, wherein said cytokeratin is cytokeratin 19.
9. The liver cell cluster of claim 3, wherein
20 said stem cell expresses desmoplakin.

- 19 -

10. The liver cell cluster of claim 6, wherein said stem cell is further characterized as expressing an antigen selected from the group consisting of laminin, desmoplakin I, cell-cell adhesion molecule (CCAM),
5 carcinoembryonic antigen (CEA), dipeptidyl peptidase-4, γ -glutamyl transpeptidase (γ GT), Very Late After Activation (VLA)-2, VLA-3, VLA-5, and VLA-6.

11. The liver cell cluster of claim 1, wherein said cluster is derived from adult liver tissue.

10 12. The cluster of claim 1, wherein said cluster is derived from a fetal or pediatric liver.

13. The liver cell cluster of claim 1, wherein said cluster is derived from human tissue.

14. The liver cell cluster of claim 1, wherein
15 said cluster is derived from rodent tissue.

15. The liver cell cluster of claim 1, wherein said stem cell differentiates into a mature functional hepatocyte or a bile duct cell.

16. The liver cell cluster of claim 1, wherein
20 said stem cell comprises heterologous DNA encoding a therapeutic protein.

- 20 -

17. The liver cell cluster of claim 16, wherein said therapeutic protein is selected from the group consisting of ornithine transcarbamylase, arginosuccinate synthetase, glutamine synthetase, glycogen synthetase, 5 glucose-6-phosphatase, succinate dehydrogenase, glucokinase, pyruvate kinase, acetyl CoA carboxylase, fatty acid synthetase, alanine aminotransferase, glutamate dehydrogenase, ferritin, low density lipoprotein (LDL) receptor, alcohol dehydrogenase, 10 albumin, transferrin, complement component C3, α_2 -macroglobulin, fibrinogen, Factor XIII:C, Factor IX, or α_1 -antitrypsin.

18. A primary liver stem cell; wherein said stem cell is 15 (a) obtained from normal liver tissue, and (b) derived from an isolated liver cell cluster comprising a hepatocyte and said stem cell.

19. The stem cell of claim 18, wherein said liver cell cluster is a cell doublet.

20 20. The stem cell of claim 18, wherein said hepatocyte and said stem cell are joined by a desmosomal junction.

21. The stem cell of claim 18, wherein said stem cell is a pre-oval cell.

25 22. The stem cell of claim 18, wherein said stem cell expresses OV6.

23. The stem cell of claim 18, wherein said stem cell expresses a bile duct cell marker.

- 21 -

24. The stem cell of claim 23, wherein said bile ductal cell marker is a cytokeratin.

25. The stem cell of claim 24, wherein said cytokeratin is cytokeratin 19.

5 26. The stem cell of claim 20, wherein said stem cell expresses desmoplakin.

27. The stem cell of claim 23, wherein said cell is further characterized as expressing an antigen selected from the group consisting of laminin, 10 desmoplakin I, CCAM, CEA, dipeptidyl peptidase-4, γ GT, VLA-2, VLA-3, VLA-5, and VLA-6.

28. The stem cell of claim 18, wherein said stem cell is derived from adult liver tissue.

29. The stem cell of claim 18, wherein said stem 15 cell is derived from a fetal or pediatric liver.

30. The stem cell of claim 18, wherein said stem cell is derived from human tissue.

31. The stem cell of claim 18, wherein said stem cell is derived from rodent tissue.

20 32. The stem cell of claim 18 wherein said stem cell differentiates into a mature functional hepatocyte or a bile duct cell.

33. The stem cell of claim 18, wherein said stem cell comprises heterologous DNA encoding a therapeutic 25 protein.

- 22 -

34. The stem cell of claim 33, wherein said therapeutic protein is selected from the group consisting of ornithine transcarbamylase, arginosuccinate synthetase, glutamine synthetase, glycogen synthetase, 5 glucose-6-phosphatase, succinate dehydrogenase, glucokinase, pyruvate kinase, acetyl CoA carboxylase, fatty acid synthetase, alanine aminotransferase, glutamate dehydrogenase, ferritin, LDL receptor, alcohol dehydrogenase, albumin, transferrin, complement component 10 C3, α_2 -macroglobulin, fibrinogen, Factor XIII:C, Factor IX, or α_1 -antitrypsin.

35. A method of obtaining a sample of isolated liver stem cells comprising

15 (a) isolating a liver cell cluster from normal liver tissue, said cluster comprising a stem cell associated with a hepatocyte;

(b) dissociating said stem cell from said hepatocyte; and

20 (c) removing said hepatocyte from said doublet to yield a sample of liver stem cells.

36. The method of claim 35, comprising the step of enriching for periportal hepatocytes associated with the biliary tree.

37. The method of claim 35, wherein said liver 25 cell cluster is a cell doublet.

38. The method of claim 35, wherein said liver cell cluster is derived from the canal of Hering of an adult liver.

- 23 -

39. The method of claim 35, further comprising selecting for expression of desmoplakin.

40. The method of claim 35, further comprising selecting for expression of OV6.

5 41. The method of claim 35, further comprising selecting for a cell which expresses an antigen selected from the group consisting of laminin, desmoplakin I, CCAM, CEA, dipeptidyl peptidase-4, γ GT, VLA-2, VLA-3, VLA-5, and VLA-6.

10 42. A liver stem cell isolated according to the method of claim 35.

43. An extracorporeal liver assist device comprising the liver cell cluster of claim 1.

15 44. An extracorporeal liver assist device comprising the liver stem cell of claim 18.

45. A method of hepatic transplantation, comprising transplanting into a mammal the liver cell cluster of claim 1.

20 46. A method of hepatic transplantation, comprising transplanting into a mammal the stem cell of claim 18.

25 47. A method of treating an inherited or acquired genetic or metabolic disease in a mammal comprising transplanting into said mammal the liver cell cluster of claim 16.

- 24 -

48. A method of treating an inherited or acquired genetic or metabolic disease in a mammal comprising transplanting into said mammal the stem cell of claim 33.

1/4

ANTIGENIC PATHWAY OF LIVER DEVELOPMENT

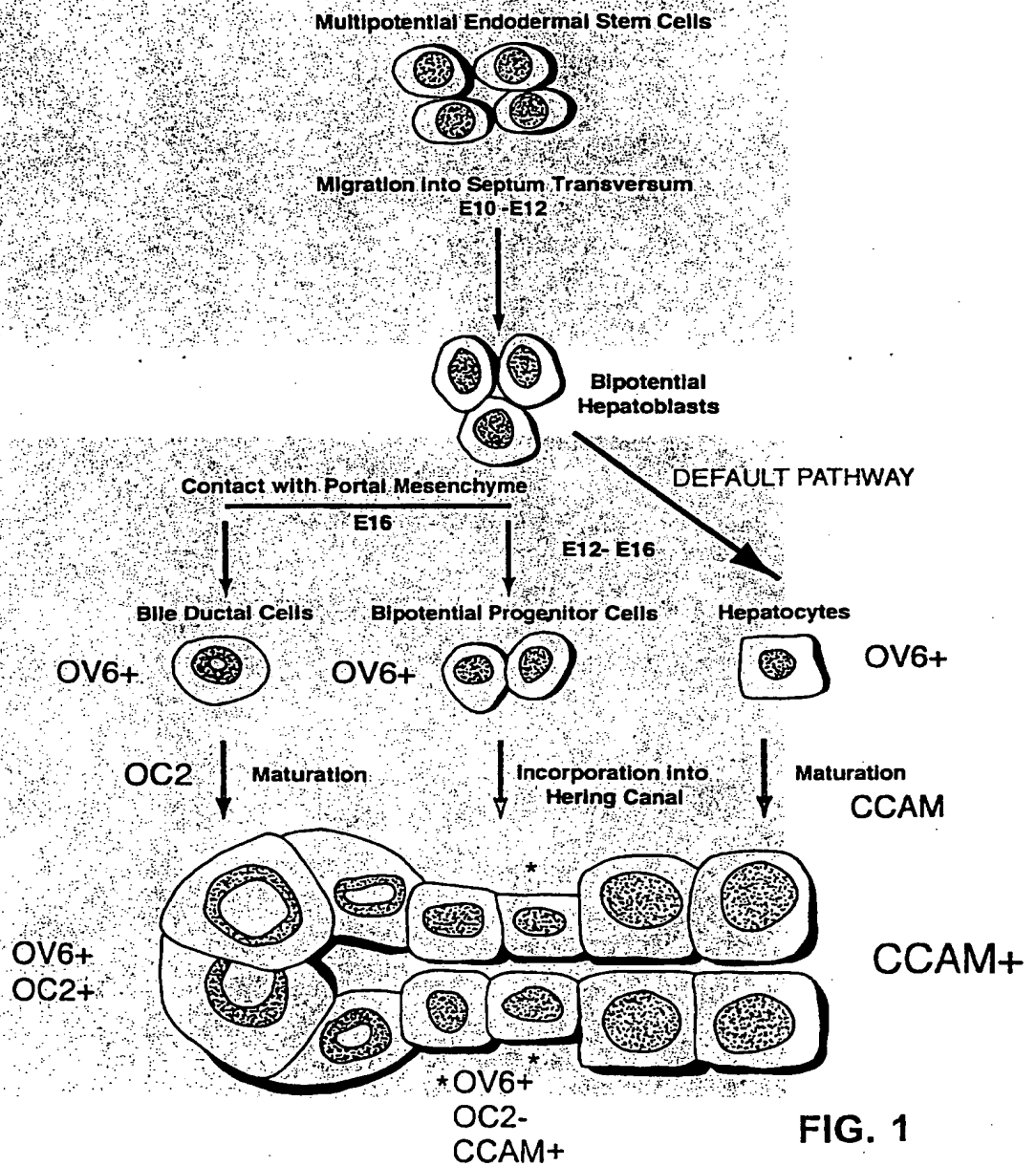
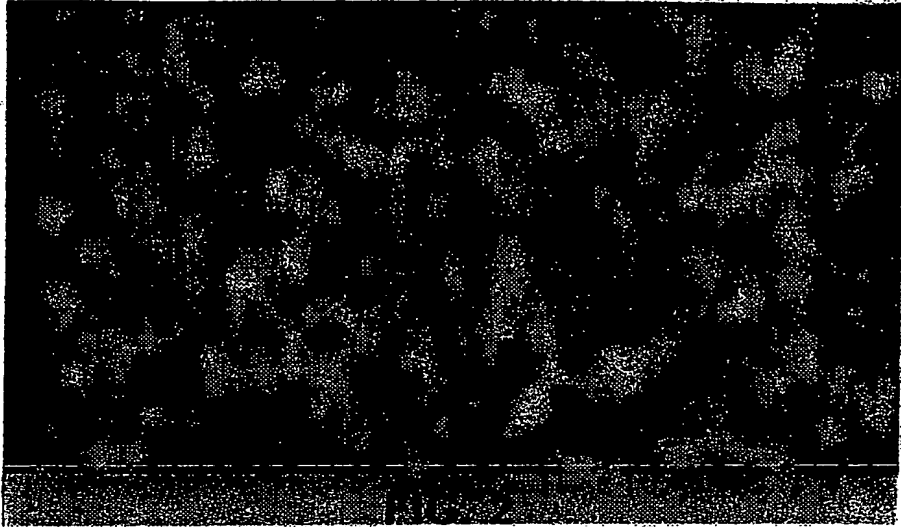
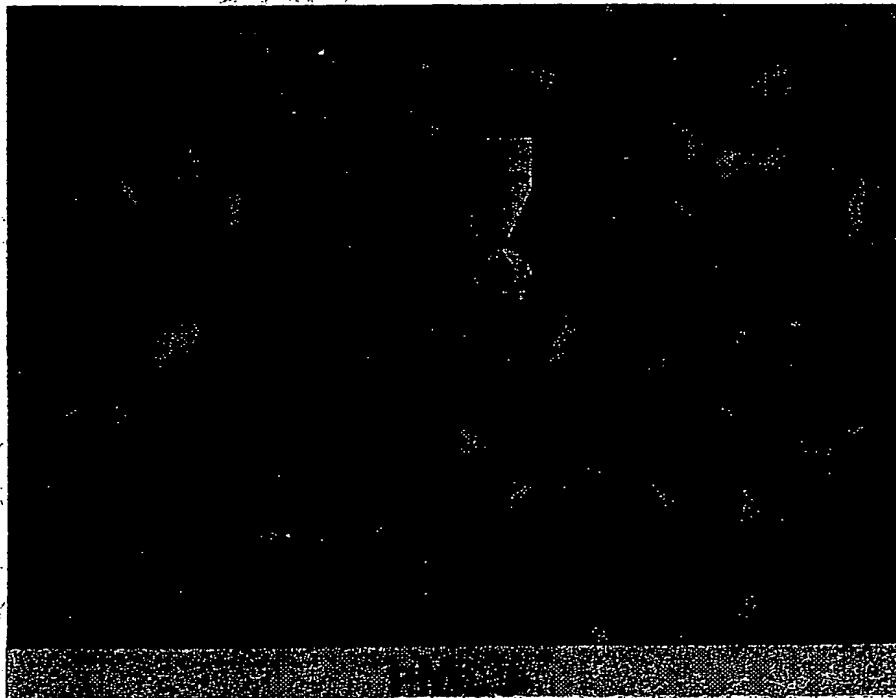


FIG. 1

2/4



RECEIVED
JAN 10 1999
FBI - NEW YORK



SUBSTITUTE SHEET (RULE 26)



FIG. 4A

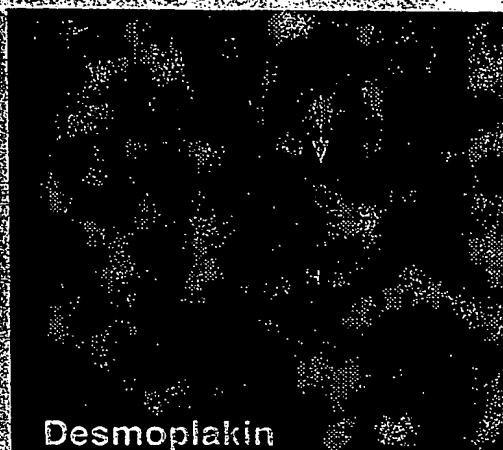
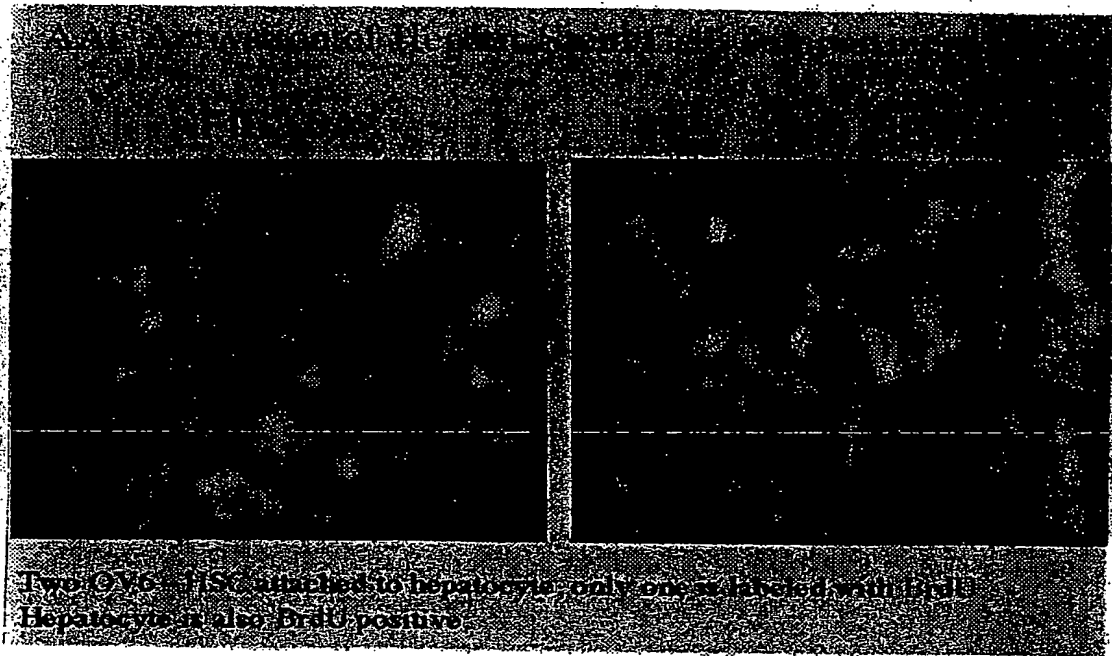


FIG. 4B

4/4



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/15625

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N5/06 C12N5/16 A61M1/14		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N A61M		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. ALISON ET AL.: "Wound healing in the liver with particular reference to stem cells" PHIL. TRANS. R. SOC. LOND. B, vol. 353, 1998, pages 877-894, XP002123293	18-34,42
Y	the whole document	1-48
X	M. AGELLI ET AL.: "Putative liver progenitor cells: conditions for long-term survival in culture" HISTOCHEMICAL JOURNAL, vol. 29, 1997, pages 205-217, XP002123294	18-34,42
Y	the whole document	1-48
-/-		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input type="checkbox"/> Patent family members are listed in annex		
* Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center; font-weight: bold;">19 November 1999</div>		Date of mailing of the international search report <div style="text-align: center; font-weight: bold;">07/12/1999</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center; font-weight: bold;">Marie, A</div>

INTERNATIONAL SEARCH REPORT

Int. Patent Application No
PCT/US 99/15625

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	S. BRILL ET AL.: "Hepatic progenitor populations in embryonic, neonatal and adult liver" P.S.E.B.M., vol. 204, 1993, pages 261-269, XP002040996	18-34,42
Y	the whole document	1-48
X	J.M. LEMIRE ET AL.: "Oval cell proliferation and the origin of small hepatocytes in liver injury induced by D-galactosamine" AMERICAN JOURNAL OF PATHOLOGY, vol. 139, no. 3, 1991, pages 535-552, XP002123295	18-34,42
Y	the whole document	1-48
Y	Y. TIAN ET AL.: "The oval shaped cell as a candidate for a liver stem cell in embryonic, neonatal and precancerous liver: identification based on morphology and pyruvate kinase isoenzyme expression" HISTOCHEM. CELL. BIOL., vol. 107, 1997, pages 243-250, XP002123296	18-34,42
Y	the whole document	1-48
X	S. SELL: "Is there a liver stem cell?" CANCER RESEARCH, vol. 50, 1990, pages 3811-3815, XP002123297	18-34,42
Y	the whole document	1-48
X	M. ALISON: "Liver stem cells: a two compartment system" CURRENT OPINION IN CELL BIOLOGY, vol. 10, 1998, pages 710-715, XP002123298	1-34,42
Y	the whole document	1-48